IN VITRO DNA SYNTHESIS AS INDICATOR OF MAMMARY EPITHELIAL CELL DIVISION: [^{14}C]THYMIDINE UPTAKE VERSUS FLOW CYTOMETRY CELL CYCLE ANALYSIS

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SUMMARY

Mammary and adipose explants from eight mid-lactation Holstein cows were co-cultured for 24 h in the presence or absence of liver explants, 1 μg/ml pituitary bovine somatotrophin, or 100 ng/ml insulinlike growth factor-I. Liver explants in the media significantly depressed DNA and protein synthesis by mammary tissue as measured by [^{14}C]-thymidine and amino acid incorporation. As measured by flow cytometry, the concentration of DNA in the G_0G_1 and G_2M cells and the percentage of cells in the G_0G_1 population of mammary tissue was also significantly depressed by liver tissue. Changes in the percentage of cells in the S and G_2M phases were not significant. Insulinlike growth factor-I in the presence of liver explants depressed protein synthesis, thymidine incorporation, and the concentration of DNA in the G_0G_1 and G_2M cells compared to control but did not affect the percentage of cells in the G_0G_1, S, or G_2M phases. Previously it was assumed that changes in [^{14}C]thymidine incorporation indicated that changes in cell division were occurring. Flow cytometry revealed that changes in DNA content of mammary cells as a result of liver or hormonal stimulation were not due to changes in cell division. Indications are that differences in cellular DNA content result from changes in the rate of amplification of individual genes responsible for milk protein synthesis.

Key words: flow cytometry; BST; IGF-I; DNA; casein; bovine.

INTRODUCTION

Bovine somatotrophin (bST) has been shown to affect milk protein and lipid synthesis by dairy cattle (16). In vitro experiments (7,13) have shown that bST acts on mammary tissue indirectly through the liver where it is thought to cause the release of insulin-like growth factor-I (IGF-I). Bauman et al. (3) hypothesized that bST/IGF-I acts on milk synthesis by redirecting the priority of nutrients toward the mammary gland. An in vitro experiment by Keys et al. (13) supported this hypothesis; bST increased lipid synthesis in mammary tissue while depressing lipid synthesis in adipose tissue incubated in the same tissue culture well with liver tissue.

It could also be hypothesized that bST and IGF-I act on milk synthesis in vivo by increasing either the number of cells or the rate at which individual cells synthesize milk. There is evidence that IGF-I stimulates the incorporation of thymidine in vitro by lactating bovine (4) and pregnant rabbit (6) mammary tissue. Duclos et al. (6) also demonstrated a concurrent increase in protein synthesis by rabbit mammary tissue with IGF-I. Houdebine et al. (12) discussed a hormonal mechanism by which prolactin stimulated casein gene transcription and casein synthesis in pregnant rabbits. New techniques such as flow cytometry can determine if changes in thymidine incorporation due to hormonal stimulation are equaled by changes in the cell cycle that represent cell division.

The objective of the current experiment was to determine if bST and IGF-I affect milk protein synthesis in bovine mammary tissue by altering the number of cells or the efficiency of individual cells. An additional objective was to compare [^{14}C]thymidine incorporation to flow cytometry as a means of measuring DNA synthesis.

MATERIALS AND METHODS

Animals and explant cultures. Mammary, liver, and adipose tissue samples were obtained from eight mid-lactation Holstein multiparous cows (226 ± 26 days of lactation, 19.5 ± 3.8 kg milk/day) at slaughter. The tissues were transported to the laboratory and converted into explants as described in (7). Tissue treatment consisted of comparing the co-culture of mammary and adipose explants to the co-culture of mammary, adipose, and liver explants. Tissue weights are presented in Table 1.

Three hormone treatments, control media; 1 μg pituitary-derived bST/ml media (1.9 IU/mg, USDA-bGH-B-1, Beltsville, MD); and 100 ng recombinant derived h-IGF-I/ml media (14 000 U/mg, Boehringer Mannheim, Indianapolis, IN) were superimposed over the tissue treatment groups. One milligram of each hormone was solubilized in 400 μl of 25 m*M* sodium carbonate (pH 9.2) and diluted to 1 μg/μl with 0.5 *M* sodium phosphate (pH 7.4) (Sigma, St. Louis, MO).

In addition to the basal medium 199 (8), 0.5 μCi/ml [^{14}C]thymidine (sp. act. = 50 mCi/mmol) was added to wells assigned to study thymidine incorporation (Amersham, Arlington Heights, IL). Also 0.5 μCi/ml [^{14}C]-leucine (sp. act. = 300 mCi/mmol), and [^{14}C]proline (sp. act. = 250 mCi/mmol) were added to wells assigned to study protein synthesis (Amersham). The tissues were incubated for 24 h under culture conditions described in (7).

Thymidine assay. Mammary explants from each well were homoge-

TABLE 1

LEAST SQUARE MEANS OF TISSUE WEIGHTS (GMS)

Tissue	Hormone			Standard Error	Probability
	Control	bST	IGF-I		
Mammary + Adipose Tissue Treatment					
Mammary	0.1433	0.1398	0.1454	0.0060	>0.85
Adipose	0.0872	0.0854	0.0846	0.0059	>0.95
Mammary + Adipose + Liver Tissue Treatment					
Mammary	0.1430	0.1407	0.1462	0.0064	>0.84
Adipose	0.1065	0.0968	0.1093	0.0100	>0.66
Liver	0.1993	0.1992	0.1952	0.0087	>0.93

Tissue Treatment				
Tissue	M + A	M + A + L	Standard Error	Probability
Mammary	0.1247	0.1260	0.0050	>0.85
Adipose	0.0735	0.1070	0.0060	<0.01

nized for 1 min in 1 ml H_2O containing 0.1 mg salmon sperm DNA and 0.1 mg thymidine (Sigma). DNA was precipitated with 10% trichloroacetic acid, centrifuged, and washed with 5% trichloroacetic acid (Sigma). The pellet was then hydrolyzed with 200 μl 60% perchloric acid (Eastman Kodak Co., Rochester, NY) and 600 μl 30% hydrogen peroxide (Fisher Scientific, Fairlawn, NJ) and incubated at 75° C for 45 min. DNA was dissolved in 2 ml ethylene glycol monoethyl ether (Sigma) and quantified by liquid scintillation counting.

Mammary epithelial cell preparation. Cells for flow cytometry were isolated from mammary explants immediately after the incubation. Explants were minced 4 times with a McIlwain tissue chopper (Mickle Lab. Eng. Co. Ltd., Gomshall, Surrey) set so that the table moved 0.25 mm for each movement of the blade. The minced material was mixed for 10 min in 3 ml ice-cold pH 7.4 phosphate buffered saline (PBS) (0.1 *M* dibasic phosphate, 0.137 *M* sodium chloride, 2.68 m*M* potassium chloride, and 1.76 m*M* monobasic potassium phosphate, Sigma) with 1% BSA (Sigma). The mixture was then filtered through 30-μm nylon mesh (Spectrum, Los Angeles, CA) to remove tissue fragments and washed with 1 ml PBS and 1% bovine serum albumin (BSA). The filtrate was centrifuged at 250 ×*g* for 5 min at 4° C and the supernatant decanted. One milliliter of PBS was added to the cell pellet and vortexed. The mixture was added slowly to 4 ml of −20° C 87.5% ethyl alcohol (Matheson, Coleman & Bell, Norwood, OH) while vortexing. The cells were incubated on ice for 20 min and stored at −20° C until assayed. Cells were demonstrated to be mammary epithelial cells by reacting with primary rabbit antibodies to bovine alpha Casein (Dr. A. J. Guidry, USDA, Beltsville, MD) and fluorescein-labeled goat anti-rabbit IgG (Kirkegaard & Perry, Gaithersburg, MD).

Flow cytometry procedures. Propidium iodide (PI) fluoresces in the red spectrum (620 to 750 nm) and intercalates between base pairs in DNA and RNA (Calbiochem, La Jolla, CA). It was used to detect DNA (20) in the mammary explant cells using an adaptation of a method described in (5). Specifically, 250 μl of the EtOH treated cells (50 000 cells) were centrifuged at 250 ×*g* for 5 min at 4° C and the EtOH decanted. Heparinized turkey blood was washed twice with PBS and fixed with EtOH. The mammary cell pellet was vibrated to loosen the pellet and 10 μl of turkey red blood cells (RBCs) containing approximately 10 000 cells and 250 μl of a 2 *N*-HCl (Fisher Scientific) solution containing 0.5% Triton X-100 (Sigma) was added. The solution was vortexed and incubated at room temperature for 30 min. Turkey RBCs have approximately 40% of the DNA content of bovine cells. There are slight within and between run electronic variations in flow cytometers. RBCs were added to provide a constant baseline that had not been altered by experimental treatment against which to measure the DNA content of bovine mammary cells (20,24).

After centrifugation, the supernatant was decanted and the cells washed with 250 μl of pH 8.5, 0.1 *N* sodium tetraborate (Fisher Scientific) to neutralize the HCl. Cells were then sequentially incubated with 50 μl of the 0.1 *M* PBS buffer containing 0.5% (vol/vol) Tween 20 (Sigma) and 1% BSA (30 min at room temperature) and 250 μl of the PBS buffer containing 5 μg PI/ml and 33 μg RNAase (Calbiochem)/ml (39° C for 15 min). The cells were then placed on ice in a refrigerator for 1 h and assayed for DNA content on an Epics profile flow cytometer (Coulter Corp., Hialeah, FL).

The Epics flow cytometer is equipped with a 488-nm argon ion laser and aspirated 100 μl containing 21 755 ± 5638 cells. Cells are released in single fashion in a hydrodynamically focused fluid stream in a flow cell and illuminated by a focused laser beam perpendicular to the flow of the cell stream. After acid denaturation of double stranded RNA and RNase digestion, PI, intercalated between base pairs of the double stranded DNA, was excited by the laser, causing it to fluoresce red. The degree of fluorescence in individual cells was measured by the flow cytometer through a 635-band pass filter in electronic channels ranging from 0 to 1023, and the number of cells in each channel recorded (20). The higher the channel number, the greater the relative DNA concentration per cell. Cells in channel number 400 would have twice the concentration of DNA per cell as cells in channel number 200, for example.

Cells were initially separated from cellular debris and doublets by means of a forward scatter vs. log side scatter histogram and were then gated to a red fluorescence histogram. Using Cytologic, a software program provided by Coulter Corp., data from the red fluorescence histogram were adjusted to 30 000 cells for each sample and analyzed for fluorescence and cell numbers in the G_0G_1, S, and G_2M phases. Factors measured include the mean DNA channel for turkey RBCs and bovine G_0G_1 and G_2M mammary cells after subtracting the mean channel for turkey RBCs to correct for electronic drift and day-to-day equipment variation; the G_2M:G_0G_1 mean channel ratio; and the percent of total cells in the G_0G_1, S, and G_2M phases.

Casein and whey protein assays. Mammary explants were homogenized in 300 μl pH 7.5 buffer/100 mg tissue (0.167 *M* tris, 1 m*M* EDTA, and 0.1 m*M* dithiotreitol, Sigma) for 30 s with a Polytron (Brinkmann Instruments, Westbury, NY) at 30% maximum power. Homogenate was centrifuged at 500 ×*g* for 1 min and cooled to harden fat plug. The fat plug was removed and the supernatant decanted into glass test tubes. Milk proteins were precipitated with 32 μl 50% trichloroacetic acid/100 μl supernatant recovered, vortexed, and allowed to sit for 5 min. The solution was centrifuged for 1 min at 1000 ×*g*, and the supernatant discarded. The precipitate was washed with 3 vol of ethyl ether (American Burdick and Jackson, Muskegon, MI), the pellet broken up, supernatant discarded, and the residual ether dried off. Then 200 μl Laemili buffer [50 m*M* tris, 0.3 m*M* EDTA, 10% β-mercapto-ethanol, 25% glycerol (Sigma), 2.5% sodium dodecyl sulfate (BioRad, Richmond, CA)] was added to the pellet, vortexed, boiled for 5 min and centrifuged at 3000 ×*g* for 4 min. Then 40 μl were added to a 15% acrylamide (BioRad) electrophoresis gel as described in (2). Purified bovine alpha S1 and buffalo beta casein (Dr. L. M. Houdebine, I.N.R.A., Jouy-en-Josas, France) and alpha lactalbumin standards were run as markers. Protein bands on dried gels were counted for [^{14}C] activity in a System 200 Imaging Scanner (Bioscan, Inc., Washington, DC).

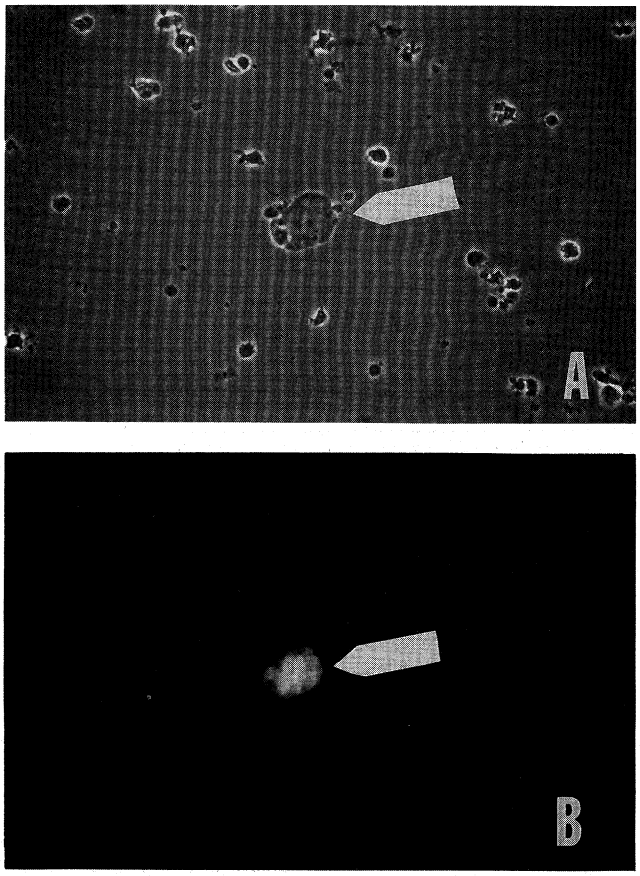

FIG. 1. Demonstration of the epithelial origin of cells prepared from bovine mammary explants by mincing with a tissue chopper. *A*, ×400 phase contrast micrograph of a bovine mammary epithelial cell (*arrow*). Debris surrounding the epithelial cell were discriminated against by the flow cytometer. *B*, ×400 micrograph of the same cell fluorescing after reacting with primary rabbit antibodies to bovine alpha casein and fluorescein labeled goat anti-rabbit IgG.

Statistical analysis. All analyses were conducted using the general linear models procedure in (19). Thymidine incorporation, protein synthesis, and the flow cytometry measures were first analyzed with a model that included cow, hormone treatment, tissue treatment, hormone × tissue treatment, and the cow × hormone × tissue treatment interaction. The cow × hormone × tissue treatment interaction was used to test all effects. Data for each tissue treatment were then analyzed separately with a model that included cow, hormone, and the cow × hormone interaction. The cow × hormone treatment interaction was used to test all effects. *t* Tests from PDIFF in (19) were used to test for differences between pairs of means, if the overall F value was significant at $P < 0.10$.

RESULTS AND DISCUSSION

The cells used in the flow cytometer analysis reacted with a fluorescein-labeled primary rabbit antibody to bovine alpha casein (Fig. 1). The results indicated that the mincing procedure used to extract cells from the mammary tissue explants produced epithelial cells. Mammary epithelial cells are the only cells in the body that produce casein. The debris surrounding the fluorescing cell in Fig. 1 were discriminated against by the flow cytometry.

G_2M phase cells have twice the diploid number of chromosomes that G_0G_1 phase cells have and twice the degree of fluorescence or a twofold higher mean DNA channel. S-phase cells are in the process of doubling their number of chromosomes (20). The G_2M:G_0G_1 mean DNA channel ratio (calculated before subtracting the turkey control mean channel) averaged 2.01 ± 0.03. Changes in the percentage of cells found in the G_0G_1, S, and G_2M phases are indicative of changes in the number of cells undergoing the process of mitosis or cell division (20). The percentage of cells in the G_0G_1 phase was found to be greater than 90% for all conditions tested (Fig. 2). Our results supported those of Saacke and Heald (18) who observed that only 10% of mammary epithelial cells were non-secretory and found few mitotic spindles by either light or electron microscopy. Similarly, Hollmann (9) found a number of binucleated cells in lactating mouse mammary tissue but observed little or no mitosis by microscopic studies. Thus it is apparent that the G_0G_1 state predominates in lactating tissue.

The addition of liver tissue to the explant cultures depressed ($P < 0.07$) the percentage of G_0G_1 mammary cells (Fig. 2) with a corresponding increase in the percentage of cells in S-phase and G_2M ($P > 0.10$). This result suggests that cell division was being increased. The addition of liver also depressed thymidine ($P < 0.01$) incorporation (Fig. 3) and the mean DNA channel of both G_0G_1 ($P < 0.01$) and G_2M ($P < 0.02$) cells as indicated by the shift of the G_0G_1 and G_2M peaks to the left on the X-axis in Fig. 4. Thus, even though there was a tendency toward cell division, the DNA content of individual cells was reduced. The addition of liver tissue also depressed ($P < 0.02$) the synthesis of casein (22 449 vs. 16 433 dpm/g mammary tissue), whey proteins (55 323 vs. 44 806 dpm/g mammary tissue), and total milk proteins (the sum of casein and whey proteins shown in Fig. 5). That the addition of liver depressed thymidine incorporation while increasing the percent S and G_2M cells, albeit not significantly, suggests that [^{14}C]-thymidine was not all being used for mitosis. The direct relationship

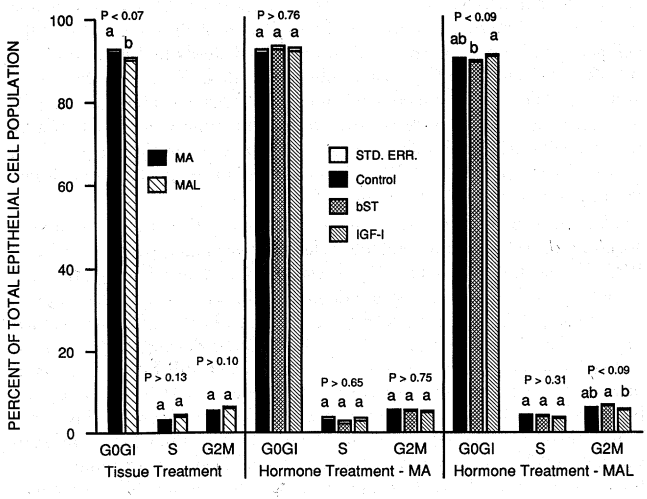

FIG. 2. Effect of tissue treatment (*MA* = mammary + adipose and *MAL* = mammary + adipose + liver) and hormone treatment within tissue treatment on the percent of total cells in the G_0G_1, S, and G_2M cell cycle phases of mammary cells prepared from mammary explants incubated for 24 h. The hormone × tissue treatment interaction was not significant ($P > 0.73$) for these effects. *Bars* within each statistical group with different superscript letters (*a,b*) are statistically different according to *t* tests from PDIFF of SAS/STAT (1987).

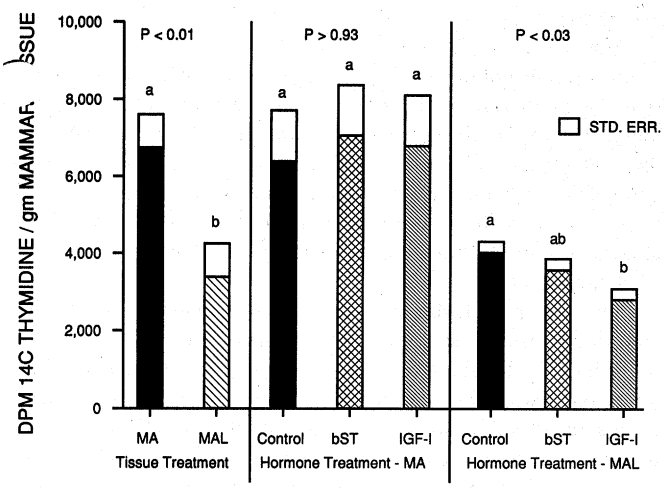

FIG. 3. Effect of tissue treatment (*MA* = mammary + adipose and *MAL* = mammary + adipose + liver) and hormone treatment within tissue treatment on the incorporation of [^{14}C]thymidine by bovine mammary tissue over 24 h. Hormone × tissue treatment interaction was not significant ($P > 0.90$) for this effect. *Bars* within each statistical group with different superscript letters (*a,b*) are statistically different according to *t* tests from PDIFF of SAS/STAT (1987).

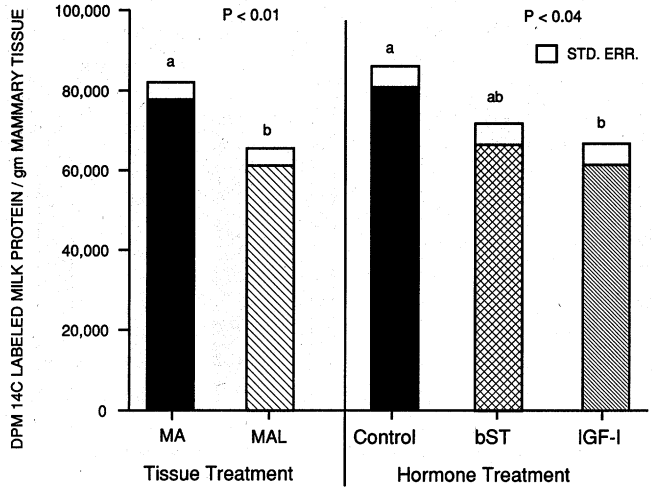

FIG. 5. Effect of tissue treatment (*MA* = mammary + adipose and *MAL* = mammary + adipose + liver) and hormone treatment across both tissue treatments on the incorporation of [^{14}C]leucine and proline into total milk proteins (casein + lactalbumin) by bovine mammary tissue incubated for 24 h. Hormone × tissue treatment interaction was not significant ($P > 0.72$) for this effect. *Bars* within each statistical group with different superscript letters (*a,b*) are statistically different according to *t* tests from PDIFF of SAS/STAT (1987).

between thymidine incorporation, the G_0G_1 and G_2M mean channel results, and protein synthesis strongly suggests that at least part of the [^{14}C]thymidine was incorporated into DNA used for transcription and protein synthesis.

Conceivably, the inclusion of liver tissue into the co-culture could have reduced the availability of nutrients to mammary tissue, thereby reducing the uptake of radiolabeled tracers. However, previous research (13,14) has demonstrated that the liver incorporated less than 2% of [^{14}C]acetate incorporated by mammary tissue during a 24-h incubation. Furthermore, 48 to 60% of the available tracer remained in the media at the end of a 24-h incubation.

Others (1,21,22), using approaches other than flow cytology, have also concluded that changes in cellular DNA content associated with changes in protein synthesis were not mitotic in nature. Sod-Moriah and Schmidt (22) suggested that the concentration of DNA per rabbit mammary cell during lactation exceeded the diploid

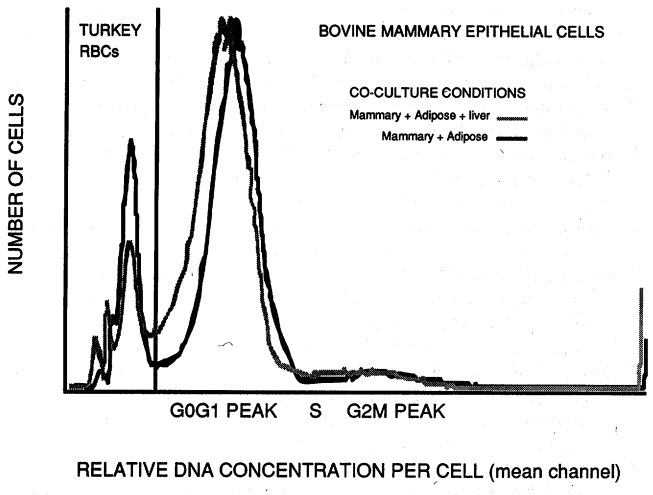

FIG. 4. Effect of tissue treatment on the mean G_0G_1 ($P < 0.01$) and G_2M ($P < 0.02$) DNA channels of bovine mammary cells prepared from mammary tissue incubated for 24 h. Mean G_0G_1 and G_2M DNA channels after subtraction of the mean turkey DNA channel for the two treatments were 175.8 ± 3.8 and 453.9 ± 7.7 without liver and 153.8 ± 3.8 and 426.4 ± 7.7 with liver, respectively.

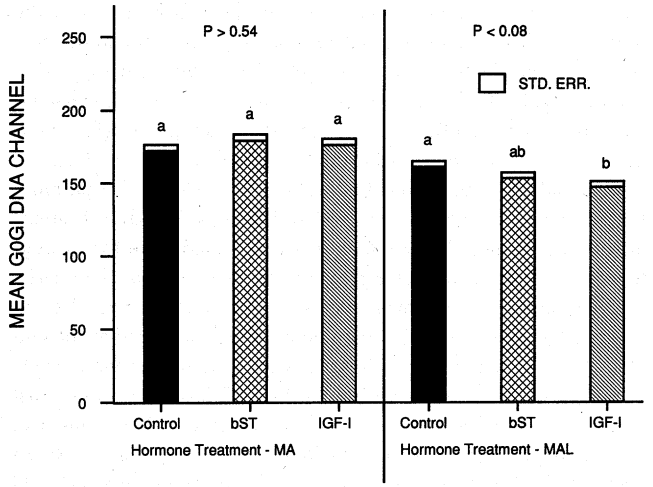

FIG. 6. Effect of hormone treatment within tissue treatment (*MA* = mammary + adipose and *MAL* = mammary + adipose + liver) on the mean G_0G_1 DNA channel of bovine mammary cells prepared from mammary tissue incubated for 24 h. Hormone × tissue treatment interaction was not significant ($P > 0.34$) for this effect. *Bars* within each statistical group with different superscript letters (*a,b*) are statistically different according to *t* tests from PDIFF of SAS/STAT (1987).

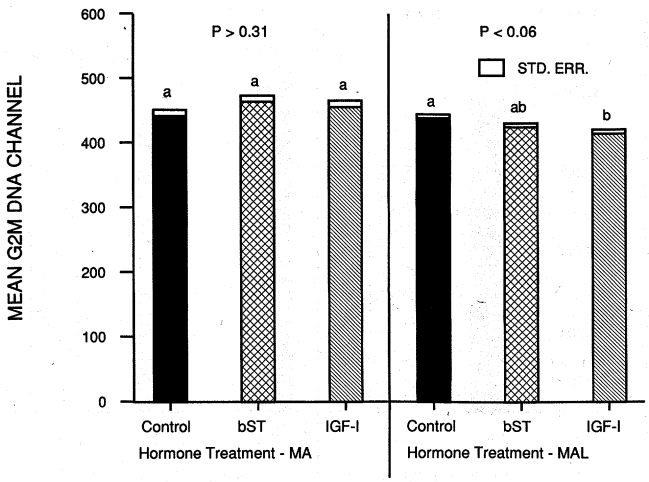

FIG. 7. Effect of hormone treatment within tissue treatment (*MA* = mammary + adipose and *MAL* = mammary + adipose + liver) on the mean G_2M DNA channel of bovine mammary cells prepared from mammary tissue incubated for 24 h. Hormone × tissue treatment interaction was not significant ($P > 0.29$) for this effect. *Bars* within each statistical group with different superscript letters (*a,b*) are statistically different according to *t* tests from PDIFF of SAS/STAT (1987).

concentration of mitotic DNA. Banerjee et al. (1) postulated that the increase in DNA seen in mouse mammary tissue was a result of an increase in copies of specific genes or "gene amplification" rather than an increase in total chromatic DNA.

Our conclusion that some of the [^{14}C]thymidine was incorporated into DNA for transcription and protein synthesis is further supported by the hormone treatment results. Thymidine (Fig. 3) incorporation ($P < 0.03$), mean G_0G_1 ($P < 0.08$), and G_2M ($P < 0.06$) channels (Figs. 6 and 7) were depressed by IGF-I in the presence of liver tissue. Total protein synthesis (Fig. 5) was depressed ($P < 0.04$) by IGF-I across liver treatments. The addition of bST had a non-significant depressing effect on the above parameters in the presence of liver tissue. Neither hormone had an effect on the percentage of cells in the G_0G_1, S, or G_2M cell populations compared to control (Fig. 2). In the absence of liver tissue, bST and IGF-I had no effect ($P > 0.31$) on the incorporation of [^{14}C]thymidine (Fig. 3) or the DNA measurements by flow cytometry (Figs. 2, 6, and 7).

Insulinlike growth factor-I had a negative effect on DNA parameters in our work whereas others (4,6,25) found that IGF-I stimulated DNA synthesis in mammary tissue. The length of the incubation period before measuring thymidine incorporation into DNA seems to be the reason for the differences between our results and those of (4,6,25). Our DNA parameters were measured at the end of a 24-h incubation. We chose this time frame because events occurring in the explants during the first 24 h of incubation most closely resemble those events in the intact animal (14). Winder et al. (25) found that maximal stimulation of DNA synthesis did not occur until after 4 to 5 days of incubation. Houdebine (10,11) demonstrated that maximal stimulation of casein and casein mRNA required 3 days incubation. DNA synthesis in (10,11,25) was very low during the first 24-h measurement period. Perhaps IGF-I causes bovine cells to dedifferentiate during the first 24 h under in vitro conditions. The deletion of amplified gene copies could be one of the first steps in the dedifferentiation process. The deletion of extrachromosomal gene copies is also seen in drug-resistant cells in the absence of stimulation (23).

The evidence of others (14,16) suggests that IGF-I may have role in both mitosis and differentiation. Monniaux and Pisselet (15) found that IGF-I stimulated progesterone synthesis, radiolabeled thymidine uptake, and cell proliferation by ovine granulosa cells of antral follicles during a 96-h incubation. Peri et al. (17), using mammary tissue from 4- to 6-mo.-old calves, found that IGF-I stimulated both thymidine uptake and lipid synthesis after 4 to 6 days incubation.

Our results point to the use of mean DNA channels in flow cytometry as a sensitive assay for studying changes in DNA synthesis occurring in cell culture as a result of hormonal stimulation. The direct relationship between the flow cytometry and radiochemical results was very dramatic and strongly indicates that [^{14}C]thymidine incorporation per se cannot be regarded as being indicative solely of cell division in bovine mammary tissue. The results suggest that some of the changes in cellular DNA content as a result of IGF-I stimulation may be due to the amplification or deamplification of specific genes responsible for protein synthesis in bovine mammary tissue.

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